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(54) Title: THERMAL TOLERANT CELLULASE FROM ACIDOTHERMUS CELLULOLYTICUS

Domain structure of GuxA



■ Signal    ▨ CBD<sub>I</sub>    ▩ CBD<sub>III</sub>    ▮ FN-III    □ Linker    ▤ Catalytic domain

(57) Abstract: The invention provides a thermal tolerant cellulase that is a member of the glycoside hydrolase family. The invention further discloses this cellulase as GuxA. GuxA has been isolated and characterized from Acidothermus cellulolyticus. The invention further provides recombinant forms of the identified GuxA. Methods of making and using GuxA polypeptides, including fusions, variants, and derivatives, are also disclosed.



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**THERMAL TOLERANT CELLULASE FROM ACIDOTHERMUS**  
**CELLULOLYTICUS**

**Government Interests**

5           The United States Government has rights in this invention under Contract No. DE-AC36-99G010337 between the United States Department of Energy and the National Renewable Energy Laboratory, a Division of the Midwest Research Institute.

**Field of the Invention**

10           The invention generally relates to a novel cellulase from *Acidothermus cellulolyticus*, GuxA. More specifically, the invention relates to purified and isolated GuxA polypeptides, nucleic acid molecules encoding the polypeptides, and processes for production and use of GuxA, as well as variants and derivatives thereof.

**Background of the Invention**

15           Plant biomass as a source of energy production can include agricultural and forestry products, associated by-products and waste, municipal solid waste, and industrial waste. In addition, over 50 million acres in the United States are currently available for biomass production, and there are a number of terrestrial and aquatic  
20   crops grown solely as a source for biomass (A Wiselogle, et al. Biomass feedstocks resources and composition. In CE Wyman, ed. Handbook on Bioethanol: Production and Utilization. Washington, DC: Taylor & Francis, 1996, pp 105-118). Biofuels produced from biomass include ethanol, methanol, biodiesel, and additives for reformulated gasoline. Biofuels are desirable because they add little, if any, net  
25   carbon dioxide to the atmosphere and because they greatly reduce ozone formation and carbon monoxide emissions as compared to the environmental output of conventional fuels. (P Bergeron. Environmental impacts of bioethanol. In CE Wyman, ed. Handbook on Bioethanol: Production and Utilization. Washington, DC: Taylor & Francis, 1996, pp 90-103).

30           Plant biomass is the most abundant source of carbohydrate in the world due to the lignocellulosic materials composing the cell walls of all higher plants. Plant cell

walls are divided into two sections, the primary and the secondary cell walls. The primary cell wall, which provides structure for expanding cells (and hence changes as the cell grows), is composed of three major polysaccharides and one group of glycoproteins. The predominant polysaccharide, and most abundant source of carbohydrates, is cellulose, while hemicellulose and pectin are also found in abundance. Cellulose is a linear beta-(1,4)-D-glucan and comprises 20% to 30% of the primary cell wall by weight. The secondary cell wall, which is produced after the cell has completed growing, also contains polysaccharides and is strengthened through polymeric lignin covalently cross-linked to hemicellulose.

Carbohydrates, and cellulose in particular, can be converted to sugars by well-known methods including acid and enzymatic hydrolysis. Enzymatic hydrolysis of cellulose requires the processing of biomass to reduce size and facilitate subsequent handling. Mild acid treatment is then used to hydrolyze part or all of the hemicellulose content of the feedstock. Finally, cellulose is converted to ethanol through the concerted action of cellulases and saccharolytic fermentation (simultaneous saccharification fermentation (SSF)). The SSF process, using the yeast *Saccharomyces cerevisiae* for example, is often incomplete, as it does not utilize the entire sugar content of the plant biomass, namely the hemicellulose fraction.

The cost of producing ethanol from biomass can be divided into three areas of expenditure: pretreatment costs, fermentation costs, and other costs. Pretreatment costs include biomass milling, pretreatment reagents, equipment maintenance, power and water, and waste neutralization and disposal. The fermentation costs can include enzymes, nutrient supplements, yeast, maintenance and scale-up, and waste disposal. Other costs include biomass purchase, transportation and storage, plant labor, plant utilities, ethanol distillation, and administration (which may include technology-use licenses). One of the major expenses incurred in SSF is the cost of the enzymes, as about one kilogram of cellulase is required to fully digest 50 kilograms of cellulose. Economical production of cellulase is also compounded by factors such as the relatively slow growth rates of cellulase-producing organisms, levels of cellulase expression, and the tendency of enzyme-dependent processes to

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partially or completely inactivate enzymes due to conditions such as elevated temperature, acidity, proteolytic degradation, and solvent degradation.

Enzymatic degradation of cellulose requires the coordinate action of at least three different types of cellulases. Such enzymes are given an Enzyme Commission (EC) designation according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Eur. J. Biochem. 264: 607-609 and 610-650, 1999). Endo- beta-(1,4)-glucanases (EC 3.2.1.4) cleave the cellulose strand randomly along its length, thus generating new chain ends. Exo- beta-(1,4)-glucanases (EC 3.2.1.91) are processive enzymes and cleave cellobiosyl units (beta-(1,4)-glucose dimers) from free ends of cellulose strands. Lastly, beta-D-glucosidases (cellobiases: EC 3.2.1.21) hydrolyze cellobiose to glucose. All three of these general activities are required for efficient and complete hydrolysis of a polymer such as cellulose to a subunit, such as the simple sugar, glucose.

Highly thermostable enzymes have been isolated from the cellulolytic thermophile *Acidothermus cellulolyticus* gen. nov., sp. nov., a bacterium originally isolated from decaying wood in an acidic, thermal pool at Yellowstone National Park. A. Mohagheghi et al., (1986) Int. J. Systematic Bacteriology, 36(3): 435-443. One cellulase enzyme produced by this organism, the endoglucanase E1, is known to display maximal activity at 75 °C to 83°C. M.P. Tucker et al. (1989), Bio/Technology, 7(8): 817-820. E1 endoglucanase has been described in U.S. Patent 5,275,944. The *A. cellulolyticus* E1 endoglucanase is an active cellulase; in combination with the exocellulase CBH I from *Trichoderma reesei*, E1 gives a high level of saccharification and contributes to a degree of synergism. Baker JO et al. (1994), Appl. Biochem. Biotechnol., 45/46: 245-256. The gene coding E1 catalytic and cellulose binding domains and linker peptide were described in U.S. Patent 5,536,655. E1 has also been expressed as a stable, active enzyme from a wide variety of hosts, including *E. coli*, *Streptomyces lividans*, *Pichia pastoris*, cotton, tobacco, and *Arabidopsis* (Dai Z, Hooker BS, Anderson DB, Thomas SR. Transgenic Res. 2000 Feb; 9(1):43-54).

There is a need within the art to generate alternative cellulase enzymes capable of assisting in the commercial-scale processing of cellulose to sugar for use

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in biofuel production. Against this backdrop the present invention has been developed.

The potential exists for the successful, commercial-scale expression of heterologous cellulase polypeptides, and in particular novel cellulase polypeptides with or without any one or more desirable properties such as thermal tolerance, and partial or complete resistance to extreme pH inactivation, proteolytic inactivation, solvent inactivation, chaotropic agent inactivation, oxidizing agent inactivation, and detergent inactivation. Such expression can occur in fungi, bacteria, and other hosts.

10

#### **Summary of the Invention**

The present invention provides GuxA, a novel member of the glycoside hydrolase (GH) family of enzymes, and in particular a thermal tolerant glycoside hydrolase useful in the degradation of cellulose. GuxA polypeptides of the invention include those having an amino acid sequence shown in SEQ ID NO:1, as well as polypeptides having substantial amino acid sequence identity to the amino acid sequence of SEQ ID NO:1 and useful fragments thereof, including, a first catalytic domain having significant sequence similarity to the GH6 family, a second catalytic domain having significant sequence similarity to the GH12 family, a first cellulose binding domain (type II) and a second cellulose binding domain (type III).

The invention also provides a polynucleotide molecule encoding GuxA polypeptides and fragments of GuxA polypeptides, for example catalytic and cellulose binding domains. Polynucleotide molecules of the invention include those molecules having a nucleic acid sequence as shown in SEQ ID NO:2; those that hybridize to the nucleic acid sequence of SEQ ID NO:2 under high stringency conditions; and those having substantial nucleic acid identity with the nucleic acid sequence of SEQ ID NO:2.

The invention includes variants and derivatives of the GuxA polypeptides, including fusion proteins. For example, fusion proteins of the invention include GuxA polypeptide fused to a heterologous protein or peptide that confers a desired function. The heterologous protein or peptide can facilitate purification, oligomerization, stabilization, or secretion of the GuxA polypeptide, for example.

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As further examples, the heterologous polypeptide can provide enhanced activity, including catalytic or binding activity, for GuxA polypeptides, where the enhancement is either additive or synergistic. A fusion protein of an embodiment of the invention can be produced, for example, from an expression construct containing  
5 a polynucleotide molecule encoding GuxA polypeptide in frame with a polynucleotide molecule for the heterologous protein. Embodiments of the invention also comprise vectors, plasmids, expression systems, host cells, and the like, containing a GuxA polynucleotide molecule. Genetic engineering methods for the production of GuxA polypeptides of embodiments of the invention include  
10 expression of a polynucleotide molecule in cell free expression systems and in cellular hosts, according to known methods.

The invention further includes compositions containing a substantially purified GuxA polypeptide of the invention and a carrier. Such compositions are administered to a biomass containing cellulose for the reduction or degradation of  
15 the cellulose.

The invention also provides reagents, compositions, and methods that are useful for analysis of GuxA activity.

These and various other features as well as advantages which characterize the present invention will be apparent from a reading of the following detailed  
20 description and a review of the associated drawings.

The following Tables 5 and 6 includes sequences used in describing embodiments of the present invention. In Table 5, the abbreviations are as follows: CD, catalytic domain; CBD\_II, carbohydrate binding domain type II; CBD\_III, carbohydrate binding domain type III; and FN-III, fibronectin domain type III. When  
25 used herein, N\* indicates a string of unknown nucleic acid units, and X\* indicates a string of unknown amino acid units, for example about 50 or more. Table 5 includes approximate start and stop information for segments, and Table 6 includes amino acid sequence data for segments.

30

Table 5. Nucleotide and polypeptide segments.

GuxA Segment	base BEGIN	base END	Length, bp	aa BEGIN No.	aa	aa END No.	aa	Length, aa	SEQ ID No. (amino acid)	SEQ ID No. (nucleotide)
Total length	1	3687	3687	1	M	1228	S	1228	1	2
Signal (potential)	1	159	159	1	M	53	A	53	3	
CD (GH6)	160	1428	1269	54	A	476	V	423	4	
CBD III	1750	2199	450	584	V	733	E	150	5	
FN-III	2266	2520	255	756	D	840	V	85	6	
CD (GH12)	2578	3270	693	860	D	1090	G	231	7	
CBD II	3382	3684	303	1128	G	1228	S	101	8	

Table 6. Gene/polypeptide segments with amino acid sequences.

SEQ ID No. (amino acid)	SEQ ID No. (nucleotide)	GuxA Segment	segment data	
			SEQ ID NO: 1 (see Table 1); SEQ ID NO: 2 (see Table 2)	
1	2	Total length Signal (potential)		
3			MERTQQSGRNCRYQRGTRMPAISKRLRAGVLAGA VSI AASIVPLAMQH PATA	
4		CD (GH6)	ATHVDNPYAGATFFVNPYWAQEVQSEAAANQTNAIIAAKMRVVSTYSTAVWMDRIAAINGVNGPGGL TTYLDAALSQQQGTTPEVIEIIVYDLPGRDCAALASNGELPATAAGLQTYETQYIDPIASILSNPK YSSLRIVTIIIEPDSLNAVNTMSIQACATAVPYYEQIEYALTKLHAIPNVYIYMDAAHSGWLGP NNASGYVQEVQKVLNASIGVNGIDGFVTNTANYTELKEPFMTATQQVGGQPVESANFYQWNPDI ADYAVDLYSRLVAAGFPSSIGMLIDTLRNGWGGNEPTGPSTADVNTFVNQSKIDLRQHRGLWCN QNGAGLGOPPPQASPTDFPNAHLDAVYVWIKPPGESDGTSAASDPTTGKKSDPMCDPTTYSYGVLTN ALPNSPIAGQWFPQAFDQLVANARPAV	
5		CBD III	YSGGLKVQYKNND SAPGNQIKPGLQLVNTGSSVDLSTVTVRYWFRDGGSTLVYNCDMAAGC GNIRASFGSVNPA TPTADTYLQLSFTGGTLAAGSTGEIQNRVNKSDWSNFTETNDYSYGTNTTFQ DWTKVTVVYVNGVLVWGT	
6		FN-III	DVTTPSVPTGLVVTGVS GSVSLAMNASTDNVGAHNVYRNGVLVGQPTVTSFTDTGLAAGTAYT YTVAADVDAAGNTSAPSTPV	
7		CD (GH12)	DCTPGPNQNGVTSVQGD EYRVQTNENWSSAQQLTINTATGAWTVSTANFSGGTGGAPATYPSIYK GCHWGNCTTKNVGMP IQISQIGSAVTSWSTTQVSSGAYDVAYDIWTNSTPTTTGQPNGTETMIWLN SRGGVQPFQSQTATGTVAGHTWNVWQGOQT SWKIIISYVLTPGATSISNLDLKAIFADAAAAGSLN TSDYLLDVEAGFEIWQGGQGLGNSFSVSVTS	
8		CBD II	GVACRAIVVNVNSDWGSGFTATVTVTNTGSRATNGHTVAVSFGGNQTVTVYWNLTALTQSGASVTATN LSYNNVTPGQSTTFGENGSYSGTNAAPTLSCTA	



### **Brief Description of the Drawings**

FIG. 1 is a schematic representation of the gene sequence and amino acid segment organization.

5        FIG 2 is a graphic representation of the glycoside hydrolase gene/protein families found in various organisms.

### **Detailed Description**

#### **Definitions:**

The following definitions are provided to facilitate understanding of certain  
10        terms used frequently herein and are not meant to limit the scope of the present disclosure:

"Amino acid" refers to any of the twenty naturally occurring amino acids as well as any modified amino acid sequences. Modifications may include natural processes such as posttranslational processing, or may include chemical  
15        modifications which are known in the art. Modifications include but are not limited to: phosphorylation, ubiquitination, acetylation, amidation, glycosylation, covalent attachment of flavin, ADP-ribosylation, cross linking, iodination, methylation, and alike.

"Antibody" refers to a Y-shaped molecule having a pair of antigen binding  
20        sites, a hinge region and a constant region. Fragments of antibodies, for example an antigen binding fragment (Fab), chimeric antibodies, antibodies having a human constant region coupled to a murine antigen binding region, and fragments thereof, as well as other well known recombinant antibodies are included in the present invention.

25        "Antisense" refers to polynucleotide sequences that are complementary to target "sense" polynucleotide sequence.

"Binding activity" refers to any activity that can be assayed by characterizing the ability of a polypeptide to bind to a substrate. The substrate can be a polymer such as cellulose or can be a complex molecule or aggregate of molecules where the  
30        entire moiety comprises at least some cellulose. Note that when used herein the

terms cellulose binding domain (CBD) and carbohydrate binding domain are used interchangeably.

"Cellulase activity" refers to any activity that can be assayed by characterizing the enzymatic activity of a cellulase. For example, cellulase activity  
5 can be assayed by determining how much reducing sugar is produced during a fixed amount of time for a set amount of enzyme (see Irwin et al., (1998) *J. Bacteriology*, 1709-1714). Other assays are well known in the art and can be substituted.

"Complementary" or "complementarity" refers to the ability of a polynucleotide in a polynucleotide molecule to form a base pair with another  
10 polynucleotide in a second polynucleotide molecule. For example, the sequence A-G-T is complementary to the sequence T-C-A. Complementarity may be partial, in which only some of the polynucleotides match according to base pairing, or complete, where all the polynucleotides match according to base pairing.

"Expression" refers to transcription and translation occurring within a host  
15 cell. The level of expression of a DNA molecule in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present within the cell or the amount of DNA molecule encoded protein produced by the host cell (Sambrook et al., 1989, *Molecular cloning: A Laboratory Manual*, 18.1-18.88).

"Fusion protein" refers to a first protein having attached a second,  
20 heterologous protein. Preferably, the heterologous protein is fused via recombinant DNA techniques, such that the first and second proteins are expressed in frame. The heterologous protein can confer a desired characteristic to the fusion protein, for example, a detection signal, enhanced stability or stabilization of the protein, facilitated oligomerization of the protein, or facilitated purification of the fusion  
25 protein. Examples of heterologous proteins useful in the fusion proteins of the invention include molecules having one or more catalytic domains of GuxA, one or more binding domains of GuxA, one or more catalytic domains of a glycoside hydrolase other than GuxA, one or more binding domains of a glycoside hydrolase other than GuxA, or any combination thereof. Further examples include  
30 immunoglobulin molecules and portions thereof, peptide tags such as histidine tag (6-His), leucine zipper, substrate targeting moieties, signal peptides, and the like.

Fusion proteins are also meant to encompass variants and derivatives of GuxA polypeptides that are generated by conventional site-directed mutagenesis and more modern techniques such as directed evolution, discussed infra.

"Genetically engineered" refers to any recombinant DNA or RNA method  
5 used to create a prokaryotic or eukaryotic host cell that expresses a protein at elevated levels, at lowered levels, or in a mutated form. In other words, the host cell has been transfected, transformed, or transduced with a recombinant polynucleotide molecule, and thereby been altered so as to cause the cell to alter expression of the desired protein. Methods and vectors for genetically engineering host cells are well  
10 known in the art; for example various techniques are illustrated in Current Protocols in Molecular Biology, Ausubel et al., eds. (Wiley & Sons, New York, 1988, and quarterly updates). Genetically engineering techniques include but are not limited to expression vectors, targeted homologous recombination and gene activation (see, for example, U.S. Patent No. 5,272,071 to Chappel) and trans activation by engineered  
15 transcription factors (see, for example, Segal et al., 1999, *Proc Natl Acad Sci USA* 96(6):2758-63).

"Glycoside hydrolase family" refers to a family of enzymes which hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Henrissat B., (1991) *Biochem. J.*, 280:309-316).  
20 Identification of a putative glycoside hydrolase family member is made based on an amino acid sequence comparison and the finding of significant sequence similarity within the putative member's catalytic domain, as compared to the catalytic domains of known family members.

"Homology" refers to a degree of complementarity between polynucleotides,  
25 having significant effect on the efficiency and strength of hybridization between polynucleotide molecules. The term also can refer to a degree of similarity between polypeptides.

"Host cell" or "host cells" refers to cells expressing a heterologous polynucleotide molecule. Host cells of the present invention express  
30 polynucleotides encoding GuxA or a fragment thereof. Examples of suitable host cells useful in the present invention include, but are not limited to, prokaryotic and

eukaryotic cells. Specific examples of such cells include bacteria of the genera *Escherichia*, *Bacillus*, and *Salmonella*, as well as members of the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*; fungi, particularly filamentous fungi such as *Trichoderma* and *Aspergillus*, *Phanerochaete chrysosporium* and other white  
5 rot fungi; also other fungi including *Fusaria*, molds, and yeast including *Saccharomyces* sp., *Pichia* sp., and *Candida* sp. and the like; plants e.g. *Arabidopsis*, cotton, barley, tobacco, potato, and aquatic plants and the like; SF9 insect cells (Summers and Smith, 1987, *Texas Agriculture Experiment Station Bulletin*, 1555), and the like. Other specific examples include mammalian cells such as human  
10 embryonic kidney cells (293 cells), Chinese hamster ovary (CHO) cells (Puck et al., 1958, *Proc. Natl. Acad. Sci. USA* 60, 1275-1281), human cervical carcinoma cells (HELA) (ATCC CCL 2), human liver cells (Hep G2) (ATCC HB8065), human breast cancer cells (MCF-7) (ATCC HTB22), human colon carcinoma cells (DLD-1) (ATCC CCL 221), Daudi cells (ATCC CRL-213), murine myeloma cells such as  
15 P3/NSI/1-Ag4-1 (ATCC TIB-18), P3X63Ag8 (ATCC TIB-9), SP2/0-Ag14 (ATCC CRL-1581) and the like.

"Hybridization" refers to the pairing of complementary polynucleotides during an annealing period. The strength of hybridization between two polynucleotide molecules is impacted by the homology between the two molecules,  
20 stringency of the conditions involved, the melting temperature of the formed hybrid and the G:C ratio within the polynucleotides.

"Identity" refers to a comparison between pairs of nucleic acid or amino acid molecules. Methods for determining sequence identity are known. See, for example, computer programs commonly employed for this purpose, such as the Gap  
25 program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wisconsin), that uses the algorithm of Smith and Waterman, 1981, *Adv. Appl. Math.*, 2: 482-489.

"Isolated" refers to a polynucleotide or polypeptide that has been separated from at least one contaminant (polynucleotide or polypeptide) with which it is  
30 normally associated. For example, an isolated polynucleotide or polypeptide is in a context or in a form that is different from that in which it is found in nature.

"Nucleic acid sequence" refers to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along a polypeptide chain. The deoxyribonucleotide sequence thus codes for the amino acid sequence.

5 "Polynucleotide" refers to a linear sequence of nucleotides. The nucleotides may be ribonucleotides, or deoxyribonucleotides, or a mixture of both. Examples of polynucleotides in the context of the present invention include single and double stranded DNA, single and double stranded RNA, and hybrid molecules having mixtures of single and double stranded DNA and RNA. The polynucleotides of the  
10 present invention may contain one or more modified nucleotides.

"Protein," "peptide," and "polypeptide" are used interchangeably to denote an amino acid polymer or a set of two or more interacting or bound amino acid polymers.

"Purify," or "purified" refers to a target protein that is free from at least 5-  
15 10% of contaminating proteins. Purification of a protein from contaminating proteins can be accomplished using known techniques, including ammonium sulfate or ethanol precipitation, acid precipitation, heat precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, size-  
20 exclusion chromatography, and lectin chromatography. Various protein purification techniques are illustrated in *Current Protocols in Molecular Biology*, Ausubel et al., eds. (Wiley & Sons, New York, 1988, and quarterly updates).

"Selectable marker" refers to a marker that identifies a cell as having undergone a recombinant DNA or RNA event. Selectable markers include, for  
25 example, genes that encode antimetabolite resistance such as the DHFR protein that confers resistance to methotrexate (Wigler et al, 1980, *Proc Natl Acad Sci USA* 77:3567; O'Hare et al., 1981, *Proc Natl Acad Sci USA*, 78:1527), the GPT protein that confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *PNAS USA*, 78:2072), the neomycin resistance marker that confers resistance to the  
30 aminoglycoside G-418 (Calberre-Garapin et al., 1981, *J Mol Biol*, 150:1), the Hygro protein that confers resistance to hygromycin (Santerre et al., 1984, *Gene* 30:147),

and the Zeocin™ resistance marker (Invitrogen). In addition, the herpes simplex virus thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes can be employed in tk<sup>-</sup>, hgp<sup>r</sup>t<sup>-</sup> and ap<sup>r</sup>t<sup>-</sup> cells, respectively.

5 "Stringency" refers to the conditions (temperature, ionic strength, solvents, etc) under which hybridization between polynucleotides occurs. A hybridization reaction conducted under high stringency conditions is one that will only occur between polynucleotide molecules that have a high degree of complementary base pairing (85% to 100% identity). Conditions for high stringency hybridization, for  
10 example, may include an overnight incubation at about 42°C for about 2.5 hours in 6 X SSC/0.1% SDS, followed by washing of the filters in 1.0 X SSC at 65°C, 0.1% SDS. A hybridization reaction conducted under moderate stringency conditions is one that will occur between polynucleotide molecules that have an intermediate degree of complementary base pairing (50% to 84% identity).

15 "Substrate targeting moiety" refers to any signal on a substrate, either naturally occurring or genetically engineered, used to target any GuxA polypeptide or fragment thereof to a substrate. Such targeting moieties include ligands that bind to a substrate structure. Examples of ligand/receptor pairs include cellulose binding domains and cellulose. Many such substrate-specific ligands are known and are  
20 useful in the present invention to target a GuxA polypeptide or fragment thereof to a substrate. A novel example is a GuxA cellulose binding domain that is used to tether other molecules to a cellulose-containing substrate such as a fabric.

"Thermal tolerant" refers to the property of withstanding partial or complete inactivation by heat and can also be described as thermal resistance or thermal  
25 stability. Although some variation exists in the literature, the following definitions can be considered typical for the optimum temperature range of stability and activity for enzymes: psychrophilic (below freezing to 10°C); mesophilic (10°C to 50°C); thermophilic (50°C to 75°C); and caldophilic (75°C to above boiling water temperature). The stability and catalytic activity of enzymes are linked  
30 characteristics, and the ways of measuring these properties vary considerably. For industrial enzymes, stability and activity are best measured under use conditions,

often in the presence of substrate. Therefore, cellulases that must act on process streams of cellulose must be able to withstand exposure up to thermophilic or even caldophilic temperatures for digestion times in excess of several hours.

In encompassing a wide variety of potential applications for embodiments of the present invention, thermal tolerance refers to the ability to function in a temperature range of from about 15°C to about 100°C. A preferred range is from about 30°C to about 80°C. A highly preferred range is from about 50°C to about 70°C. For example, a protein that can function at about 45°C is considered in the preferred range even though it may be susceptible to partial or complete inactivation at temperatures in a range above about 45°C and less than about 80°C. For polypeptides derived from organisms such as *Acidothermus*, the desirable property of thermal tolerance among is often accompanied by other desirable characteristics such as: resistance to extreme pH degradation, resistance to solvent degradation, resistance to proteolytic degradation, resistance to detergent degradation, resistance to oxidizing agent degradation, resistance to chaotropic agent degradation, and resistance to general degradation. Cowan DA in Danson MJ et al. (1992) The Archaeobacteria, Biochemistry and Biotechnology at 149-159, University Press, Cambridge, ISBN 1855780100. Here 'resistance' is intended to include any partial or complete level of residual activity. When a polypeptide is described as thermal tolerant it is understood that any one, more than one, or none of these other desirable properties can be present.

"Variant", as used herein, means a polynucleotide or polypeptide molecule that differs from a reference molecule. Variants can include nucleotide changes that result in amino acid substitutions, deletions, fusions, or truncations in the resulting variant polypeptide when compared to the reference polypeptide.

"Vector," "extra-chromosomal vector" or "expression vector" refers to a first polynucleotide molecule, usually double-stranded, which may have inserted into it a second polynucleotide molecule, for example a foreign or heterologous polynucleotide. The heterologous polynucleotide molecule may or may not be naturally found in the host cell, and may be, for example, one or more additional copy of the heterologous polynucleotide naturally present in the host genome. The

vector is adapted for transporting the foreign polynucleotide molecule into a suitable host cell. Once in the host cell, the vector may be capable of integrating into the host cell chromosomes. The vector may optionally contain additional elements for selecting cells containing the integrated polynucleotide molecule as well as elements  
5 to promote transcription of mRNA from transfected DNA. Examples of vectors useful in the methods of the present invention include, but are not limited to, plasmids, bacteriophages, cosmids, retroviruses, and artificial chromosomes.

Within the application, unless otherwise stated, the techniques utilized may be found in any of several well-known references, such as: *Molecular Cloning: A Laboratory Manual* (Sambrook et al. (1989) Molecular cloning: A Laboratory  
10 Manual), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991 Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutscher, 3d., (1990) Academic Press, Inc.), *PCR Protocols: A Guide to Methods and Applications* (Innis et al.  
15 (1990) Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique*, 2<sup>nd</sup> ed. (R.I. Freshney (1987) Liss, Inc., New York, NY), and *Gene Transfer and Expression Protocols*, pp 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.).

## 20 **O-Glycoside Hydrolases:**

Glycoside hydrolases are a large and diverse family of enzymes that hydrolyse the glycosidic bond between two carbohydrate moieties or between a carbohydrate and a non-carbohydrate moiety (See FIG. 2). Glycoside hydrolase enzymes are classified into glycoside hydrolase (GH) families based on significant  
25 amino acid similarities within their catalytic domains. Enzymes having related catalytic domains are grouped together within a family, (Henrissat et al., (1991) supra, and Henrissat et al. (1996), *Biochem. J.* 316:695-696), where the underlying classification provides a direct relationship between the GH domain amino acid sequence and how a GH domain will fold. This information ultimately provides a  
30 common mechanism for how the enzyme will hydrolyse the glycosidic bond within a



substrate, *i.e.*, either by a retaining mechanism or inverting mechanism (Henrissat, B, (1991) *supra*).

Cellulases belong to the GH family of enzymes. Cellulases are produced by a variety of bacteria and fungi to degrade the  $\beta$ -1,4 glycosidic bond of cellulose and to so produce successively smaller fragments of cellulose and ultimately produce glucose. At present, cellulases are found within are at least 11 different GH families. Three different types of cellulase enzyme activities have been identified within these GH families: exo-acting cellulases which cleave successive disaccharide units from the non-reducing ends of a cellulose chain; endo-acting cellulases which randomly cleave successive disaccharide units within the cellulose chain; and  $\beta$ -glucosidases which cleave successive disaccharide units to glucose (J. W. Deacon, (1997) *Modern Mycology*, 3rd Ed., ISBN: 0-632-03077-1, 97-98).

Many cellulases are characterized by having a multiple domain unit within their overall structure, a GH or catalytic domain is joined to a cellulose-binding domain (CBD) by a glycosylated linker peptide (see FIG. 1) (Koivula et al., (1996) *Protein Expression and Purification* 8:391-400). As noted above, cellulases do not belong to any one family of GH domains, but rather have been identified within at least 11 different GH families to date. The CBD type domain increases the concentration of the enzyme on the substrate, in this case cellulose, and the linker peptide provides flexibility for both larger domains.

Conversion of cellulose to glucose is an essential step in the production of ethanol or other biofuels from biomass. Cellulases are an important component of this process, where approximately one kilogram of cellulase can digest fifty kilograms of cellulose. Within this process, thermostable cellulases have taken precedent, due to their ability to function at elevated temperatures and under other conditions including pH extremes, solvent presence, detergent presence, proteolysis, etc. (see Cowan DA (1992), *supra*).

Highly thermostable cellulase enzymes are secreted by the cellulolytic thermophile *Acidothermus cellulolyticus* (U.S. Patent Nos. 5,275,944 and 5,110,735). This bacterium was originally isolated from decaying wood in an acidic, thermal pool at Yellowstone National Park and deposited with the American Type

Culture Collection (ATCC 43068) (Mohagheghi et al., (1986) *Int. J. System. Bacteriol.*, 36:435-443).

Recently, a thermostable cellulase, E1 endoglucanase, was identified and characterized from *Acidothermus cellulolyticus* (U.S. Patent No. 5,536,655). The E1  
5 endoglucanase has maximal activity between 75 and 83°C and is active to a pH well below 5. Thermostable cellulase, and E1 endoglucanase, are useful in the conversion of biomass to biofuels, and in particular, are useful in the conversion of cellulose to glucose. Conversion of biomass to biofuel represents an extremely important alternative fuel source that is more environmentally friendly than  
10 conventional fuels, and provides a use, in some cases, for waste products.

#### **GuxA:**

As described more fully in the Examples below, GuxA, a novel thermostable cellulase, has now been identified and characterized. The predicted amino acid  
15 sequence of GuxA (SEQ ID NO: 1) has an organization characteristic of a cellulase enzyme. GuxA contains two catalytic domain-linker domain-cellulose binding domain units, separated from each other by a centrally located fibronectin domain. In particular, a first unit is located at the N-terminal end of the protein and includes a GH6 domain (amino acids 54-476)-linker-CBD<sub>III</sub> (amino acids 584-733), and a  
20 second unit, that includes a GH12 domain, is located at the C-terminal end of the protein (amino acids 860-1090)-linker-CBD<sub>II</sub> (amino acids 1128-1228). As discussed in more detail below, significant amino acid similarity of GuxA to other cellulases identifies GuxA as a cellulase.

GuxA, as noted above, has two catalytic domains, identified as belonging to  
25 the GH6 and GH12 families. The GH6 domain family includes a number of cellobiohydrolases, for example, exocellobiohydrolase A isolated from *Cellulomonas fimi*, and exoglucanase E3 isolated from *Thermobifida fusca*. The GH6 members degrade substrate using an inverting mechanism. The GH12 domain family includes a number of endoglucanases, for example, endo-1,4-glucanase  
30 isolated from *Streptomyces lividans*, and endo-1,4-glucanase S cellulase 12A

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isolated from *Streptomyces* sp. 11AG8. The GH12 members degrade substrate using a retaining mechanism.

Being a member of the GH6 and GH12 family of proteins identifies GuxA as potentially having both exoglucanase and endoglucanase activity. In addition, the  
5 predicted amino acid sequence (SEQ ID NO: 2) indicates that CBD type II and CBD type III domains are present as characterized by Tomme P. et al. (1995), in Enzymatic Degradation of Insoluble Polysaccharides (Saddler JN & Penner M, eds.), at 142-163, American Chemical Society, Washington. See also Tomme, P. & Claeyssens, M. (1989) FEBS Lett. 243, 239-243; Gilkes, N.R et al., (1988)  
10 J.Biol.Chem. 263, 10401-10407.

GuxA is also a thermostable cellulase as it is produced by the thermophile *Acidothermus cellulolyticus*. As discussed, GuxA polypeptides can have other desirable characteristics (see Cowan DA (1992), supra). Like other members of the cellulase family, and in particular thermostable cellulases, GuxA polypeptides are  
15 useful in the conversion of biomass to biofuels and biofuel additives, and in particular, biofuels from cellulose. It is envisioned that GuxA polypeptides could be used for other purposes, for example in detergents, pulp and paper processing, food and feed processing, and in textile processes. GuxA polypeptides can be used alone or in combination with one or more other cellulases or glycoside hydrolases to  
20 perform the uses described herein or known within the relevant art, all of which are within the scope of the present disclosure.

#### **GuxA Polypeptides:**

GuxA polypeptides of the invention include isolated polypeptides having an  
25 amino acid sequence as shown below in Example 1; Table 1 and in SEQ ID NO:1, as well as variants and derivatives, including fragments, having substantial identity to the amino acid sequence of SEQ ID NO:1 and that retain any of the functional activities of GuxA. GuxA polypeptide activity can be determined, for example, by subjecting the variant, derivative, or fragment to a substrate binding assay or a  
30 cellulase activity assay such as those described in Irwin D et al., J. Bacteriology 180(7): 1709-1714 (April 1998).

**Table 1. GuxA amino acid sequence. (SEQ ID NO: 1)**

MERTQQSGRNCRYQRGTTTRMPAISKRLRAGVLGAVSIAASIVPLAMQHPIAATHVDNPY  
 AGATFFVNPYWAQEVQSEANQTNATLAAKMRVVSTYSTAVWMDRIAANGVNGGPGLTT  
 5 YLDAALSQQQGTTPVEIVYDLPGRDCAALASNGELPATAAGLQTYETQYIDPIASILSNPK  
 YSSLRIVTIIEPDLPNAVNTNMSIQACATAVPYYEQGIEYALTKLHAIPNVYIYMDAAHSGWL  
 GWPNNASGYVQEVQKVLNASIGVNGIDGFVTNTANYTPLKEPFMTATQQVGGQPVESANFY  
 QWNPDIIDEADYAVDLYSRLVAAGFPSSIGMLIDTLRNGWGGPNEPTGPSTATDVNTFVNQSK  
 10 IDLRQHRGLWCNQNGAGLGQPPQASPTDFPNAHLDAYVWIKPPGESDGTSAASDPTTGKKS  
 DPMCDPTYTTSYGVLTNALPNSPIAGQWFPQFDQLVANARPAVPTSTSSSPPPPPSPSPSP  
 PSPSPSPSSSPSPSPSSSPSPSPSPSPSSSPSPSPSPSPSPSSSPSPSPSSSPSPSPSPSP  
 SPSSSPSPSPTSSPVSGGLKVQYKNNDAPGDNQIKPGLQLVNTGSSSVDLSTVTVRYWFTRD  
 GGSSTLVYNCDDWAAMGCGNIRASFGSVNPATPTADTYLQLSFTGGTLAAGGSTGEIQNRVN  
 15 KSDWSNFTETNDYSYGTNTTFQDWTKVTVYVNGVLVWGTEPSGTSPSPSPSPSPSPSPGG  
 DVTPPSVPTGLVVTGVSGSSVSLAWNASTDNVGVVAHYNVYRNGVLVGQPTVTSFTDTGLAA  
 GTAYTYTVAAVDAAGNTSAPSTPVTATTTSPSPSPPTPTGTTVTDCTPGPNQNGVTSVQGDEY  
 RVQTNENWNSSAQCLTINTATGAWTVSTANFSGGTGGAPATYPSIYKGCCHWNCCTTKNVG  
 MPIQISQIGSAVTSWSTTQVSSGAYDVAYDIWTNSTPTTTGQPNGTEIMIWLNSRGGVQPFGS  
 20 QTATGVTVAGHTWNVWQGGQTSWKIISYVLTPGATSISNLDLKAIFADAAARGSLNTSDYLL  
 DVEAGFEIWQGGQGLGSNSFSVSTSGTSSPTSPSPPTPTPSPTPTPSPTPTSPSPPTSSPSSGV  
 ACRATYVVNSDWGSGFTATVTVTNTGSRATNGWTVAVWSFGGNQVTNTYWNALTQSGAS  
 VTATNLSYNNVIQPGQSTTFGFNGSYSGTNAAPTLSTAS

25 As listed and described in Tables 1 and 5, the isolated GuxA polypeptide  
 includes an N-terminal hydrophobic region that functions as a signal peptide, having  
 an amino acid sequence that begins with Met1 and extends to about Ala53; a first  
 catalytic domain having significant sequence similarity to a GH6 family domain that  
 begins with about Ala54 and extends to about Val476, a cellulose binding domain type  
 30 III region that begins with about Val584 and extends to about Glu733, a fibronectin  
 type III domain that begins with about Asp756 and extends to about Val840, a second  
 catalytic domain having significant sequence similarity to a GH12 family domain that  
 begins with about Asp860 and extends to about Gly1090, and a cellulose binding  
 domain type II that begins with about Gly1128 and extends to about Ser1228. Variants  
 35 and derivatives of GuxA include, for example, GuxA polypeptides modified by  
 covalent or aggregative conjugation with other chemical moieties, such as glycosyl  
 groups, polyethylene glycol (PEG) groups, lipids, phosphate, acetyl groups, and the  
 like.

The amino acid sequence of GuxA polypeptides of the invention is in some  
 40 embodiments about 60% identical, and in other embodiments about 70% identical,  
 or in some embodiments about 90% identical, to the GuxA amino acid sequence

shown above in Table 1 and SEQ ID NO: 1. The percentage identity, also termed homology (see definition above) can be readily determined, for example, by comparing the two polypeptide sequences using any of the computer programs commonly employed for this purpose, such as the Gap program (Wisconsin Sequence Analysis  
5 Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wisconsin), which uses the algorithm of Smith and Waterman, 1981, *Adv. Appl. Math.* 2: 482-489.

Variants and derivatives of the GuxA polypeptide may further include, for example, fusion proteins formed of a GuxA polypeptide and a heterologous  
10 polypeptide. Preferred heterologous polypeptides include those that facilitate purification, oligomerization, stability, or secretion of the GuxA polypeptides.

GuxA polypeptide fragments may include, but are not limited to, the polypeptide sequences listed in Table 5, SEQ ID NOS: 3, 4, 5, 6, 7 and 8.

GuxA polypeptide variants and derivatives, as used in the description of the  
15 invention, can contain conservatively substituted amino acids, meaning that one or more amino acid can be replaced by an amino acid that does not alter the secondary and/or tertiary structure of the polypeptide. Such substitutions can include the replacement of an amino acid, by a residue having similar physicochemical properties, such as substituting one aliphatic residue (Ile, Val, Leu, or Ala) for another, or  
20 substitutions between basic residues Lys and Arg, acidic residues Glu and Asp, amide residues Gln and Asn, hydroxyl residues Ser and Tyr, or aromatic residues Phe and Tyr. Phenotypically silent amino acid exchanges are described more fully in Bowie *et al.*, 1990, *Science* 247:1306-1310. In addition, functional GuxA polypeptide variants include those having amino acid substitutions, deletions, or additions to the amino acid  
25 sequence outside functional regions of the protein, for example, outside the catalytic and cellulose binding domains. These would include, for example, the various linker sequences that connect functional domains as defined herein.

The GuxA polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. The polypeptides may be  
30 recovered and purified from recombinant cell cultures by known methods, including, for example, ammonium sulfate or ethanol precipitation, anion or cation exchange

chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography. Preferably, high performance liquid chromatography (HPLC) is employed for purification.

5           Another embodiment of the invention provides for a form of GuxA polypeptide and polypeptides that are recombinant polypeptides expressed by suitable hosts. Furthermore, the hosts can simultaneously produce other cellulases such that a mixture is produced comprising a GuxA polypeptide and one or more other cellulases. Such a mixture can be effective in crude fermentation processing or other industrial  
10       processing.

          GuxA polypeptides can be fused to heterologous polypeptides to facilitate purification. Many available heterologous peptides (peptide tags) allow selective binding of the fusion protein to a binding partner. Non-limiting examples of peptide tags include 6-His, thioredoxin, hemagglutinin, GST, and the OmpA signal sequence  
15       tag. A binding partner that recognizes and binds to the heterologous peptide can be any molecule or compound, including metal ions (for example, metal affinity columns), antibodies, antibody fragments, or any protein or peptide that preferentially binds the heterologous peptide to permit purification of the fusion protein.

          GuxA polypeptides can be modified to facilitate formation of GuxA oligomers.  
20       For example, GuxA polypeptides can be fused to peptide moieties that promote oligomerization, such as leucine zippers and certain antibody fragment polypeptides, for example, Fc polypeptides. Techniques for preparing these fusion proteins are known, and are described, for example, in WO 99/31241 and in Cosman et.al., 2001 *Immunity* 14:123-133. Fusion to an Fc polypeptide offers the additional advantage of  
25       facilitating purification by affinity chromatography over Protein A or Protein G columns. Fusion to a leucine-zipper (LZ), for example, a repetitive heptad repeat, often with four or five leucine residues interspersed with other amino acids, is described in Landschultz et al., 1988, *Science*, 240:1759.

          It is also envisioned that an expanded set of variants and derivatives of GuxA  
30       polynucleotides and/or polypeptides can be generated to select for useful molecules, where such expansion is achieved not only by conventional methods such as site-

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directed mutagenesis (SDM) but also by more modern techniques, either independently or in combination.

Site-directed-mutagenesis is considered an informational approach to protein engineering and can rely on high-resolution crystallographic structures of target proteins and some stratagem for specific amino acid changes (Van Den Burg, B.; Vriend, G.; Veltman, O.R.; Venema, G.; Eijssink, V.G.H. *Proc. Nat. Acad. Sci. U.S.* 1998, 95, 2056-2060). For example, modification of the amino acid sequence of GuxA polypeptides can be accomplished as is known in the art, such as by introducing mutations at particular locations by oligonucleotide-directed mutagenesis (Walder et al., 1986, *Gene*, 42:133; Bauer et al., 1985, *Gene* 37:73; Craik, 1985, *BioTechniques*, 12:19; Smith et al., 1981, *Genetic Engineering: Principles and Methods*, Plenum Press; and U.S. Patent No. 4,518,584 and U.S. Patent No. 4,737,462). SDM technology can also employ the recent advent of computational methods for identifying site-specific changes for a variety of protein engineering objectives (Hellings, H.W. *Nature Structural Biol.* 1998, 5, 525-527).

The more modern techniques include, but are not limited to, non-informational mutagenesis techniques (referred to generically as "directed evolution"). Directed evolution, in conjunction with high-throughput screening, allows testing of statistically meaningful variations in protein conformation (Arnold, F.H. *Nature Biotechnol.* 1998, 16, 617-618). Directed evolution technology can include diversification methods similar to that described by Cramer et al. (1998, *Nature* 391: 288-291), site-saturation mutagenesis, staggered extension process (StEP) (Zhao, H.; Giver, L.; Shao, Z.; Affholter, J.A.; Arnold, F.H. *Nature Biotechnol.* 1998, 16, 258-262), and DNA synthesis/reassembly (U.S. Patent 5,965,408).

Fragments of the GuxA polypeptide can be used, for example, to generate specific anti-GuxA antibodies. Using known selection techniques, specific epitopes can be selected and used to generate monoclonal or polyclonal antibodies. Such antibodies have utility in the assay of GuxA activity as well as in purifying recombinant GuxA polypeptides from genetically engineered host cells.

**GuxA Polynucleotides:**

The invention also provides polynucleotide molecules encoding the GuxA polypeptides discussed above. GuxA polynucleotide molecules of the invention include polynucleotide molecules having the nucleic acid sequence shown in Table 2 and SEQ ID NO: 2, polynucleotide molecules that hybridize to the nucleic acid sequence of Table 2 and SEQ ID NO: 2 under high stringency hybridization conditions (for example, 42°, 2.5 hr., 6X SCC, 0.1%SDS); and polynucleotide molecules having substantial nucleic acid sequence identity with the nucleic acid sequence of Table 2 and SEQ ID NO: 2, particularly with those nucleic acids encoding the two catalytic domains, GH6 (from amino acid 54 to 476) and GH12 (from amino acid 860 to 1090), the cellulose binding domain III (from amino acid 584 to 733) and cellulose binding domain II (from amino acid 1128 to 1228).

**Table 2. GuxA nucleotide sequence. (SEQ ID NO: 2)**

15 ATGGAGCGAACCCAACAATCCGGACGGAAGTGCAGGTACCAGAGAGGAACGACACGAA  
 TGCCCGCCATCTCAAAACGGCTGCGAGCCGGCGTCTCGCCGGGGCGGTGAGCATCGCA  
 GCCTCCATCGTGCCGCTGGCGATGCAGCATCTGCCATCGCCGCGACGCACGTCGACAAT  
 CCCTATGCGGGAGCGACCTTCTTCGTCAACCCGTACTGGGCGCAAGAAGTACAGAGCGA  
 AGCGGCGAACCAGACCAATGCCACTCTCGCAGCGAAAATGCGCGTCGTTTCCACATATTC  
 20 GACGGCCGTCTGGATGGACCGCATCGCTGCGATCAACGGCGTCAACGGCGGACCCGGCT  
 TGACGACATATCTGGACGCCGCCCTCTCCAGCAGCAGGGAACCAACCCCTGAAGTCATTG  
 AGATTGTCTACGATCTGCCGGGACGCGACTGCGCGGCGCTCGCCTCCAACGGCGAA  
 CTGCCCGCTACGGCAGCAGGTTTGAGACCTATGAAACGCAGTACATCGATCCGATTGCG  
 AGTATCCTGAGCAATCCGAAGTACTCCAGCCTGCGGATCGTGACGATCATTGAGCCGGA  
 25 CTCGCTGCCAAACGCGGTACCAATATGAGCATTCAAGCGTGTGCAACGGCGGTGCCGT  
 ATTACGAGCAAGGCATCGAGTACGCGCTCACGAAATTGCACGCCATTCCGAACGTGTAC  
 ATCTACATGGACGCCGCCCACTCCGGCTGGCTTGGGTGGCCCAATAATGCCAGCGGATAC  
 GTACAGGAAGTCCAGAAGGTCCTCAACGCGAGCATCGGGGTCAACGGCATCGACGGCTT  
 CGTCACCAACACGGCGAATTACACGCCGTTGAAGGAGCCGTTTCATGACCGCCACCCAGC  
 30 AGGTCGGCGGACAGCCGGTGGAGTCGGCGAATTTCTACCAAGTGAATCCTGACATCGAC  
 GAAGCCGACTACGCGGTTGACTTGTAAGTCTCGCGGCTCGTCGCCGCTGGCTTTCCAAGCAGC  
 ATCGGCATGCTCATCGACACCTTACGCAACGGTTGGGGTGGTCCGAACGAACCAACAGG  
 CCCGAGCACCGCGACCGATGTCAACACCTTCGTCAACCAAGTCAAGATTGACCTTCGGCA  
 GCACCGCGGCTGTGGTGAACCAAGCGTGCAGGCTCGGCCAGCCGCCGAGGCAA  
 35 GCCGACGGACTTCCGAACGCGCACCTCGACGCGTATGTCTGGATCAAGCCGCCGGGT  
 GAGTCGGACGGCACAAGCGCTGCGAGCGATCCGACAAGTGGCAAGAAGTCGGACCCCAT  
 GTGCGACCCGACGTACACGACGTCGTACGGGGTACTGACCAACGCGTTACCGAAGTCCC  
 CGATCGCCGCGCCAGTGGTTCCCGGCGCAGTTTGACCAAGCTTGTGCGGAACGACGCGCA  
 GCGGTGCCGACGTCGACCAAGTCTGAGCCCGCGCCTCCGCCGCCGAGTCCGTCGGCTTCG  
 40 CCGAGTCCGAGCCCGAGTCCGAGCCCGAGCAGCTCGCCATCGCCGTCGCCGTCTCCGAGC  
 TCGAGCCCGTCTCCGTCGCCGAGCCCGAGTCCGAGCCCGAGTAGCTCGCCGTCGCCGTCT  
 CCGAGCTCGAGCCCGTCTCCGTCGCCGAGCCCGAGTCCGAGCCCGAGTAGCTCGCCGTCG  
 CCGTCTCCGAGCTCGAGCCCGTCTCCGTCGCCGAGCCCGAGTCCGAGCCCGAGTAGCTCG  
 CCGTCGCCGTCTCCGACGTCGTCGCCGGTGTGGGTGGGCTGAAGGTGCAGTACAAGAA



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CAATGATTCGGCGCCGGGTGATAACCAGATCAAACCGGGTCTCCAGTTGGTGAATACCG  
 GGTCGTCGTCGGTGGATTTGTCGACGGTGACGGTGCGGTACTGGTTCACCCGGGATGGT  
 GGGTCGTCGACACTGGTGTACAACTGTGACTGGGCGGCGATGGGGTGTGGGAATATCCG  
 CGCCTCGTTCGGCTCGGTGAACCCGGCGACGCCGACGGCGGACACCTACCTGCAGTTGTC  
 5 GTTCACTGGTGGAACGTTGGCCGCTGGTGGGTGACGGGTGAGATTCAAAACCGGGTGA  
 ATAAGAGTGACTGGTCAATTTCACCGAGACCAATGACTACTCGTATGGGACGAACACC  
 ACCTTCCAGGACTGGACGAAGGTGACGGTGTACGTCAACGGCGTGTGGTGTGGGGGAC  
 TGAACCGTCCGGCACCAGCCCCAGCCCCACACCATCCCCGAGCCCGAGCCCGAGCCCGA  
 GCCCGGGTGGGGATGTGACGCCGCCGAGTGTGCCGACCGGCTTGGTGGTGACGGGGGTG  
 10 AGTGGGTGTCGTCGGTGTCTGTGGCGTGGAAATGCGTCGACGGATAACGTGGGGGTGGCGCA  
 TTACAACGTGTACCGCAACGGGGTGTGGTGGGGCCAGCCGACGGTGACCTCGTTACCG  
 ACACGGGTTTGGCCGCGGGAACCGCGTACACCTACACGGTGGCCGCGGTGGACGCTGCG  
 GGTAACACCTCCGCCCATCCACCCCGTCACCGCCACCACCACGAGTCCAGCCCCAGC  
 CCCACGCCGACGGGGACCACGGTCACCGACTGCACGCCCGGTCTTAACAGAATGGTGT  
 15 GACCAGCGTGCAGGGCGACGAATACCGGGTGCAGACCAATGAGTGGAAATTCGTCGGCCC  
 AGCAGTGCCTCACCATCAATACCGCGACCGGTGCCTGGACGGTGAGCACTGCGAACTTC  
 AGCGGTGGGACCGGCGGTGCGCCCGGACGTATCCGTCGATCTACAAGGGGTGCCACTG  
 GGGCAACTGCACCACGAAGAACGTGCGGGATGCCGATTGAGATCAGTCAGATTGGTTCGG  
 CTGTGACGTCGTGGAGTACGACGCAGGTGTCGTGCGGCGCGTATGACGTGGCCTACGAC  
 20 ATTTGGACGAACAGTACCCCAACGACAACCGGTGAGCCAAACGGTACCGAAATCATGAT  
 TTGGCTGAATTCGCGTGGTGGGGTGCAGCCGTTCCGGTTCGAGACAGCGACGGGTGTGA  
 CGGTCGCTGGTCACACGTGGAATGTCTGGCAGGGTCAGCAGACCTCGTGGAAGATTATT  
 TCCTACGTCTTGACCCCCGGTGCGACGTGATCAGTAATCTGGATTTGAAGGCGATTTTC  
 GCGGACGCCGCGGCACGCGGGTCGCTCAACACCTCCGATTACCTGCTCGACGTTGAGGC  
 25 CGGGTTTGAGATCTGGCAAGGTGGTCAGGGCCTGGGCAGCAACTCGTTCAGCGTCTCCG  
 TGACGAGCGGCACGTCCAGCCCCGACACCGAGCCCGAGCCCGACGCCGACACCGAGCCCG  
 ACGCCGACACCGTCTCCGAGCCCCGACCCCGTCGCCGAGTCCGACCAGCTCGCCGTCGTG  
 TCGGGTGTGGCGTGCCGGGCGACGTATGTGGTGAATAGTGATTGGGGTTCTGGGTTTAC  
 GGCGACGGTGACGGTGACGAATACCGGGAGCCGGGCGACGAACGGGTGGACGGTGGCG  
 30 TGGTCGTTTGGTGGGAATCAGACGGTCACGAACACTGGAACACTGCGTTGACCCAATC  
 AGGTGCATCGGTGACGGCGACGAACCTGAGTTACAACAACGTGATCCAACCGGGTCAGT  
 CGACCACCTTCGATTCAACGGAAGTTACTCAGGAACAAACGCCGCGCCGACGCTCAGC  
 TGCACAGCCAGCTGA

35 The GuxA polynucleotide molecules of the invention are preferably isolated  
 molecules encoding the GuxA polypeptide having an amino acid sequence as shown in  
 Table 1 and SEQ ID NO: 1, as well as derivatives, variants, and useful fragments of  
 the GuxA polynucleotide. The GuxA polynucleotide sequence can include deletions,  
 substitutions, or additions to the nucleic acid sequence of Table 2 and SEQ ID NO: 2.

40 The GuxA polynucleotide molecule of the invention can be cDNA, chemically  
 synthesized DNA, DNA amplified by PCR, RNA, or combinations thereof. Due to  
 the degeneracy of the genetic code, two DNA sequences may differ and yet encode  
 identical amino acid sequences. The present invention thus provides an isolated  
 polynucleotide molecule having a GuxA nucleic acid sequence encoding GuxA  
 45 polypeptide, where the nucleic acid sequenc encodes a polypeptide having the

complete amino acid sequences as shown in Table 1 and SEQ ID NO: 1, or variants, derivatives, and fragments thereof.

The GuxA polynucleotides of the invention have a nucleic acid sequence that is in some embodiments about 60% identical to the nucleic acid sequence shown in Table 2 and SEQ ID NO: 2, in some embodiments about 70% identical to the nucleic acid sequence shown in Table 2 and SEQ ID NO: 2, and in other embodiments about 90% identical to the nucleic acid sequence shown in Table 2 and SEQ ID NO: 2. Nucleic acid sequence identity is determined by known methods, for example by aligning two sequences in a software program such as the BLAST program (Altschul, S.F et al. (1990) J. Mol. Biol. 215:403-410, from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>)).

The GuxA polynucleotide molecules of the invention also include isolated polynucleotide molecules having a nucleic acid sequence that hybridizes under high stringency conditions (as defined above) to a the nucleic acid sequence shown in Table 2 and SEQ ID NO: 2. Hybridization of the polynucleotide is to at least about 15 contiguous nucleotides, or at least about 20 contiguous nucleotides, and in other embodiments at least about 30 contiguous nucleotides, and in still other embodiments at least about 100 contiguous nucleotides of the nucleic acid sequence shown in Table 2 and SEQ ID NO: 2.

Useful fragments of the GuxA-encoding polynucleotide molecules described herein, include probes and primers. Such probes and primers can be used, for example, in PCR methods to amplify and detect the presence of GuxA polynucleotides *in vitro*, as well as in Southern and Northern blots for analysis of GuxA. Cells expressing the GuxA polynucleotide molecules of the invention can also be identified by the use of such probes. Methods for the production and use of such primers and probes are known. For PCR, 5' and 3' primers corresponding to a region at the termini of the GuxA polynucleotide molecule can be employed to isolate and amplify the GuxA polynucleotide using conventional techniques.

Other useful fragments of the GuxA polynucleotides include antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence capable of

binding to a target GuxA mRNA (using a sense strand), or DNA (using an antisense strand) sequence.

#### **Vectors and Host Cells:**

5           The present invention also provides vectors containing the polynucleotide molecules of the invention, as well as host cells transformed with such vectors. Any of the polynucleotide molecules of the invention may be contained in a vector, which generally includes a selectable marker and an origin of replication, for propagation in a host. The vectors further include suitable transcriptional or translational regulatory  
10 sequences, such as those derived from a mammalian, microbial, viral, or insect genes, operably linked to the GuxA polynucleotide molecule. Examples of such regulatory sequences include transcriptional promoters, operators, or enhancers, mRNA ribosomal binding sites, and appropriate sequences which control transcription and translation. Nucleotide sequences are operably linked when the regulatory sequence  
15 functionally relates to the DNA encoding the target protein. Thus, a promoter nucleotide sequence is operably linked to a GuxA DNA sequence if the promoter nucleotide sequence directs the transcription of the GuxA sequence.

Selection of suitable vectors for the cloning of GuxA polynucleotide molecules encoding the target GuxA polypeptides of this invention will depend upon the host cell  
20 in which the vector will be transformed, and, where applicable, the host cell from which the target polypeptide is to be expressed. Suitable host cells for expression of GuxA polypeptides include prokaryotes, yeast, and higher eukaryotic cells, each of which is discussed below.

The GuxA polypeptides to be expressed in such host cells may also be fusion  
25 proteins that include regions from heterologous proteins. As discussed above, such regions may be included to allow, for example, secretion, improved stability, or facilitated purification of the GuxA polypeptide. For example, a nucleic acid sequence encoding an appropriate signal peptide can be incorporated into an expression vector. A nucleic acid sequence encoding a signal peptide (secretory leader) may be fused  
30 in-frame to the GuxA sequence so that GuxA is translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host

cell promotes extracellular secretion of the GuxA polypeptide. Preferably, the signal sequence will be cleaved from the GuxA polypeptide upon secretion of GuxA from the cell. Non-limiting examples of signal sequences that can be used in practicing the invention include the yeast I-factor and the honeybee melatin leader in Sf9 insect cells.

5           Suitable host cells for expression of target polypeptides of the invention include prokaryotes, yeast, and higher eukaryotic cells. Suitable prokaryotic hosts to be used for the expression of these polypeptides include bacteria of the genera *Escherichia*, *Bacillus*, and *Salmonella*, as well as members of the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. For expression in prokaryotic cells,  
10           for example, in *E. coli*, the polynucleotide molecule encoding GuxA polypeptide preferably includes an N-terminal methionine residue to facilitate expression of the recombinant polypeptide. The N-terminal Met may optionally be cleaved from the expressed polypeptide.

            Expression vectors for use in prokaryotic hosts generally comprise one or more  
15           phenotypic selectable marker genes. Such genes encode, for example, a protein that confers antibiotic resistance or that supplies an auxotrophic requirement. A wide variety of such vectors are readily available from commercial sources. Examples include pSPORT vectors, pGEM vectors (Promega, Madison, WI), pPROEX vectors (LTI, Bethesda, MD), Bluescript vectors (Stratagene), and pQE vectors (Qiagen).

20           GuxA can also be expressed in yeast host cells from genera including *Saccharomyces*, *Pichia*, and *Kluveromyces*. Preferred yeast hosts are *S. cerevisiae* and *P. pastoris*. Yeast vectors will often contain an origin of replication sequence from a 2T yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a  
25           selectable marker gene. Vectors replicable in both yeast and *E. coli* (termed shuttle vectors) may also be used. In addition to the above-mentioned features of yeast vectors, a shuttle vector will also include sequences for replication and selection in *E. coli*. Direct secretion of the target polypeptides expressed in yeast hosts may be accomplished by the inclusion of nucleotide sequence encoding the yeast I-factor  
30           leader sequence at the 5' end of the GuxA-encoding nucleotide sequence.

Insect host cell culture systems can also be used for the expression of GuxA polypeptides. The target polypeptides of the invention are preferably expressed using a baculovirus expression system, as described, for example, in the review by Luckow and Summers, 1988 *Bio/Technology* 6:47.

5           The choice of a suitable expression vector for expression of GuxA polypeptides of the invention will depend upon the host cell to be used. Examples of suitable expression vectors for *E. coli* include pET, pUC, and similar vectors as is known in the art. Preferred vectors for expression of the GuxA polypeptides include the shuttle plasmid pIJ702 for *Streptomyces lividans*, pGAPZalpha-A, B, C and  
10 pPICZalpha-A, B, C (Invitrogen) for *Pichia pastoris*, and pFE-1 and pFE-2 for filamentous fungi and similar vectors as is known in the art.

          Modification of a GuxA polynucleotide molecule to facilitate insertion into a particular vector (for example, by modifying restriction sites), ease of use in a particular expression system or host (for example, using preferred host codons), and  
15 the like, are known and are contemplated for use in the invention. Genetic engineering methods for the production of GuxA polypeptides include the expression of the polynucleotide molecules in cell free expression systems, in cellular hosts, in tissues, and in animal models, according to known methods.

## 20   **Compositions**

          The invention provides compositions containing a substantially purified GuxA polypeptide of the invention and an acceptable carrier. Such compositions are administered to biomass, for example, to degrade the cellulose in the biomass into simpler carbohydrate units and ultimately, to sugars. These released sugars from the  
25 cellulose are converted into ethanol by any number of different catalysts. Such compositions may also be included in detergents for removal, for example, of cellulose containing stains within fabrics, or compositions used in the pulp and paper industry, to address conditions associated with cellulose content. Compositions of the present invention can be used in stonewashing jeans such as is well known in the  
30 art. Compositions can be used in the biopolishing of cellulosic fabrics, such as cotton, linen, rayon and Lyocell.

The invention provides pharmaceutical compositions containing a substantially purified GuxA polypeptide of the invention and if necessary a pharmaceutically acceptable carrier. Such pharmaceutical compositions are administered to cells, tissues, or patients, for example, to aid in delivery or targeting of other pharmaceutical compositions. For example, GuxA polypeptides may be used where carbohydrate-mediated liposomal interactions are involved with target cells. Vyas SP et al. (2001), *J. Pharmacy & Pharmaceutical Sciences* May-Aug 4(2): 138-58.

The invention also provides reagents, compositions, and methods that are useful for analysis of GuxA activity and for the analysis of cellulose breakdown.

Compositions of the present invention may also include other known cellulases, and preferably, other known thermal tolerant cellulases for enhanced treatment of cellulose.

#### 15   **Antibodies**

The polypeptides of the present invention, in whole or in part, may be used to raise polyclonal and monoclonal antibodies that are useful in purifying GuxA, or detecting GuxA polypeptide expression, as well as a reagent tool for characterizing the molecular actions of the GuxA polypeptide. Preferably, a peptide containing a unique epitope of the GuxA polypeptide is used in preparation of antibodies, using conventional techniques. Methods for the selection of peptide epitopes and production of antibodies are known. See, for example, *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), 1988 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet et al. (eds.), 1980 Plenum Press, New York.

#### **Assays**

Agents that modify, for example, increase or decrease, GuxA hydrolysis or degradation of cellulose can be identified, for example, by assay of GuxA cellulase activity and/or analysis of GuxA binding to a cellulose substrate. Incubation of cellulose in the presence of GuxA and in the presence or absence of a test agent and

correlation of cellulase activity or cellulose binding permits screening of such agents. For example, cellulase activity and binding assays may be performed in a manner similar to those described in Irwin et al., *J. Bacteriology* 180(7): 1709-1714 (April 1998).

5           The GuxA stimulated activity is determined in the presence and absence of a test agent and then compared. A lower GuxA activated test activity in the presence of the test agent, than in the absence of the test agent, indicates that the test agent has decreased the activity of the GuxA. A higher GuxA activated test activity in the presence of the test agent than in the absence of the test agent indicates that the test  
10       agent has increased the activity of the GuxA. Stimulators and inhibitors of GuxA may be used to augment, inhibit, or modify GuxA mediated activity, and therefore may have potential industrial uses as well as potential use in the further elucidation of GuxA's molecular actions.

## 15       **Therapeutic Applications**

          The GuxA polypeptides of the invention are effective in adding in delivery or targeting of other pharmaceutical compositions within a host. For example, GuxA polypeptides may be used where carbohydrate-mediated liposomal interactions are involved with target cells. Vyas SP et al. (2001), *J. Pharm Pharm Sci* May-Aug  
20       4(2): 138-58.

          GuxA polynucleotides and polypeptides, including vectors expressing GuxA, of the invention can be formulated as pharmaceutical compositions and administered to a host, preferably mammalian host, including a human patient, in a variety of forms adapted to the chosen route of administration. The compounds are preferably  
25       administered in combination with a pharmaceutically acceptable carrier, and may be combined with or conjugated to specific delivery agents, including targeting antibodies and/or cytokines.

          GuxA can be administered by known techniques, such as orally, parentally (including subcutaneous injection, intravenous, intramuscular, intrasternal or  
30       infusion techniques), by inhalation spray, topically, by absorption through a mucous membrane, or rectally, in dosage unit formulations containing conventional non-

toxic pharmaceutically acceptable carriers, adjuvants or vehicles. Pharmaceutical compositions of the invention can be in the form of suspensions or tablets suitable for oral administration, nasal sprays, creams, sterile injectable preparations, such as sterile injectable aqueous or oleaginous suspensions or suppositories.

5 For oral administration as a suspension, the compositions can be prepared according to techniques well-known in the art of pharmaceutical formulation. The compositions can contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners or flavoring agents. As immediate release tablets, the compositions  
10 can contain microcrystalline cellulose, starch, magnesium stearate and lactose or other excipients, binders, extenders, disintegrants, diluents and lubricants known in the art.

For administration by inhalation or aerosol, the compositions can be prepared according to techniques well-known in the art of pharmaceutical formulation. The  
15 compositions can be prepared as solutions in saline, using benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons or other solubilizing or dispersing agents known in the art.

For administration as injectable solutions or suspensions, the compositions can be formulated according to techniques well-known in the art, using suitable  
20 dispersing or wetting and suspending agents, such as sterile oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

For rectal administration as suppositories, the compositions can be prepared by mixing with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters or polyethylene glycols, which are solid at ambient temperatures, but  
25 liquefy or dissolve in the rectal cavity to release the drug.

Preferred administration routes include orally, parenterally, as well as intravenous, intramuscular or subcutaneous routes. More preferably, the compounds of the present invention are administered parenterally, i.e., intravenously or intraperitoneally, by infusion or injection.

30 Solutions or suspensions of the compounds can be prepared in water, isotonic saline (PBS) and optionally mixed with a nontoxic surfactant. Dispersions may also



be prepared in glycerol, liquid polyethylene, glycols, DNA, vegetable oils, triacetin and mixtures thereof. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage form suitable for injection or infusion use can include sterile, aqueous solutions or dispersions or sterile powders comprising an active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol such as glycerol, propylene glycol, or liquid polyethylene glycols and the like, vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size, in the case of dispersion, or by the use of nontoxic surfactants. The prevention of the action of microorganisms can be accomplished by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, buffers, or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the inclusion in the composition of agents delaying absorption--for example, aluminum monostearate hydrogels and gelatin.

Sterile injectable solutions are prepared by incorporating the compounds in the required amount in the appropriate solvent with various other ingredients as enumerated above and, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

### Industrial Applications

The GuxA polypeptides of the invention are effective cellulases. In the methods of the invention, the cellulose degrading effects of GuxA are achieved by treating biomass at a ratio of about 1 to about 50 of GuxA:biomass. GuxA may be  
5 used under extreme conditions, for example, elevated temperatures and acidic pH. Treated biomass is degraded into simpler forms of carbohydrates, and in some cases glucose, which is then used in the formation of ethanol or other industrial chemicals, as is known in the art. Other methods are envisioned to be within the scope of the present invention, including methods for treating fabrics to remove cellulose-  
10 containing stains and other methods already discussed. GuxA polypeptides can be used in any known application currently utilizing a cellulase, all of which are within the scope of the present invention.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of  
15 illustration and are not intended as limiting.

### EXAMPLES

#### Example 1: Molecular Cloning of GuxA

Genomic DNA was isolated from *Acidothermus cellulolyticus* and purified  
20 by banding on cesium chloride gradients. Genomic DNA was partially digested with Sau 3A and separated on agarose gels. DNA fragments in the range of 9-20 kilobase pairs were isolated from the gels. This purified Sau 3A digested genomic DNA was ligated into the Bam HI acceptor site of purified EMBL3 lambda phage arms (Clontech, San Diego, Calif.). Phage DNA was packaged according to the  
25 manufacturer's specifications and plated with *E. coli* LE392 in top agar which contained the soluble cellulose analog, carboxymethylcellulose (CMC). The plates were incubated overnight (12-24 hours) to allow transfection, bacterial growth, and plaque formation. Plates were stained with Congo Red followed by destaining with 1 M NaCl. Lambda plaques harboring endoglucanase clones showed up as unstained  
30 plaques on a red background.

Lambda clones which screened positive on CMC-Congo Red plates were purified by successive rounds of picking, plating and screening. Individual phage isolates were named SL-1, SL-2, SL-3 and SL-4. Subsequent subcloning efforts employed the SL-3 clone which contained an approximately 14.2 kb fragment of *A. cellulolyticus* genomic DNA.

Template DNA was constructed using a 9 kb BamHI fragment obtained from the 14.2 kb lambda clone SL3 prepared from *Acidothermus cellulolyticus* genomic DNA. The 9-kb BamHI fragment from SL3 was subcloned into pDR540 to generate a plasmid NREL501. NREL501 was first sequenced by the primer walking method as is known in the art. NREL501 was then subcloned into pUC19 using restriction enzymes PstI and EcoRI and transformed into *E. coli* XL1-blue (Stratagene, La Jolla, California) for the production of template DNA for sequencing. Each subclone was sequenced from both forward and reverse directions. DNA for sequencing was prepared from an overnight growth in 500 mL LB broth using a megaprep DNA purification kit from Promega. The template DNA was PEG precipitated and suspended in de-ionized water and adjusted to a final concentration of 0.25 mg/mL. Custom primers were designed by reading upstream known sequence and selecting segments of an appropriate length to function, as is well known in the art. Primers for cycle sequencing were synthesized at the Macromolecular Resources facility located at Colorado State University in Fort Collins, Colorado. Typically the sequencing primers were 26-30 nucleotides in length, but were sometimes longer or shorter to accommodate a melting temperature appropriate for cycle sequencing. The sequencing primers were diluted in de-ionized water, the concentration measured using UV absorbance at 260 nm, and then adjusted to a final concentration of 5 pmol/ $\mu$ L. Templates and sequencing primers were shipped to the Iowa State University DNA Sequencing facility at Ames, Iowa for sequencing using standard chemistries for cycle sequencing. In many cases, regions of the template that sequenced poorly using the standard protocols and dye terminators were repeated with the addition of 2  $\mu$ L DMSO and by using nucleotides optimized for the sequencing of high GC content DNA. The high frequency of reoccurring small domains (ie, CBDs and linkers) with high sequence similarity caused initial

difficulties in sequence assignments which were only resolved through extensive review of the data and repeat analyses.

Sequencing data from primer walking and subclones were assembled together to verify that all SL3 regions had been sequenced from both strands. An open reading frames (ORF) was found in the 9-kb BamHI fragment, C-terminal of E1 (patent 5,536,655), termed GuxA.

An ORF of about 3687 bp [SEQ ID NO: 2], including a stop codon, and deduced amino acid sequence [SEQ ID NO:1] are shown in Tables 1 and 2. The amino acid sequence predicted by SEQ ID NO: 1 was determined to have significant homology to known cellulases, as shown below in Example 2 and in Tables 3 and 4. The amino acid sequence represents a novel member of the family of proteins with cellulase activity. Due to the source of isolation from the thermophilic organism *Acidothermus*, GuxA is a novel member of cellulases with properties including thermal tolerance. It is also known that thermal tolerant enzymes may have other properties (see definition above).

#### **Example 2: GuxA includes a GH6 catalytic domain**

Sequence alignments and comparisons of the amino acid sequences of the *Acidothermus cellulolyticus* GuxA first catalytic domain (aa 54 to 476), *Cellulomonas fimi* CBHA (beta-(1,4) exocellobiohydrolase) and *Thermobifida fusca* E3 (beta-(1,4) exocellulase) polypeptides were prepared, using the ClustalW program (Thompson J.D et al. (1994), Nucleic Acids Res. 22:4673-4680 from EMBL European Bioinformatics Institute website (<http://www.ebi.ac.uk/>).

An examination of the amino acid sequence alignment of the GH6 domains indicates that the amino acid sequence of the GuxA catalytic domain is homologous to the amino acid sequences of known GH6 family catalytic domains for *C. fimi* CBHA and *T. fusca* E3 (See Table 3). In Table 3, the notations are as follows: an asterisk "\*" indicates identical or conserved residues in all sequences in the alignment; a colon ":" indicates conserved substitutions; a period "." indicates semi-conserved substitutions; and a hyphen "-" indicates a gap in the sequence. The amino acid sequence predicted for the GuxA GH6 domain is approximately 55%

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identical to the *C. fimi* CBHA GH6 domain and approximately 48% identical to the *T. fusca* E3 GH6 domain, indicating that the GuxA first catalytic domain is a member of the GH6 family (Henrissat et al. (1991), supra).

**5 Table 3. Multiple amino acid sequence alignment of a GuxA first catalytic domain and polypeptides with Glycoside Hydrolase Family 6 catalytic domains.**

Multialignment of related Glycoside Hydrolase Family 6 catalytic domain

GH6\_Ace: Acidothermus cellulolyticus GuxA catalytic domain GH6

10 CBHA\_Cfi: Cellulomonas fimi CBHA (beta-1,4-exocellobiohydrolase). GeneBank Acc. # AAC36898

E3\_Tfu: Thermobifida fusca E3 (beta-1,4-exocellulase). GeneBank Acc. # U18978

15	GH6_Ace CBHA_Cfi E3_Tfu	-ATHVDNPFYAGATFFVNPYWAQEVQSEANQTN-ATLAAKMRVSTYSTAVVMDRIAAN APVHVDNPFYAGAVQYVNPFWAASVNAAAGRSADPALAAKMRVAGQPTAVVMDRISAIT PGGFTNPPTNPGEKVDNPFEGAKLYVNPVW-SAKAAAEFGGSANESTAVWLDRIGAIIE *                   **                   :                   *                   *                   *                   *
20	GH6_Ace CBHA_Cfi E3_Tfu	GVN---GGPGLTTYLDAAALSQQQGT-TPEVIEIVYIDLPGRDCAALASNGELPATAAGL GNA----DGNGLKHFHLDNAVQQKAAGVPLVFNLVYIDLPGRDCAALASNGELPATDAGL GNDSPPTGSMGLRDHLEBAVRQSGGD--PLTIQVVIYNLPGRDCAALASNGELGPDE--L *                   .                   **                   *:                   *                   *                   *                   *                   *                   *                   *
25	GH6_Ace CBHA_Cfi E3_Tfu	QTYETQYIDPIASILSN-PKYSSLRIVTIIIEPDSLNAVITNMSIQACATAVPYIEQ---- ARYKSEYIDPIADLLDN-PEYESIRIAATIEPDSLNLTTNISEPAQQQAAPYRQ---- DRYKSEYIDPIADIMWDFADYENLRIVAIIEIDSLPNLVTVNGGNGTELCAVMKQNGGY *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *
30	GH6_Ace CBHA_Cfi E3_Tfu	--GIEYALTKLHAIPNVYIYMDAAHSGWLGWPNNASGYVQEVQKVLN-ASIGVNGIDGFV --GVKYALDKLHAIPNVYNYIDIGHSGWLGWDSNAGPSATLFAEVAKSTTAGFASIDGFV VNGVGVALRKLGEIPNVYNYIDAAHHGWIGWDSNFGPSVDIFYEANASGSTVDYVHGFI *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *
35	GH6_Ace CBHA_Cfi E3_Tfu	TNTANYTPLKEPFMT-ATQQVGGQPVESANFYQWNPDIIDEADYAVDLYSRLVAAGFPSSI SDVANTTPLEELLSDSLITINNTPIRSSKFYEWNFDFEIDYTAHMRLLVAAGFPSSI SNTANYSATVEPYLD-VNGTVNGQLIRQSKWVDWNQYVDELSFVQDLRQALIAKGFSDI :                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *
40	GH6_Ace CBHA_Cfi E3_Tfu	GMLIDTLRNGWGGPNEPTGPSTATDVNTFVNQSKIDLRQHRGLWCNQGAGLGQPPQASP GMLVDTSRNGWGGPNRPTSTASTDVNAYVDANRVDRVHRGAWCNPLGAGIGRPFPEATP GMLIDTSRNGWGGPNRPTGPSSSTDLNTYVDESRIDRRIHGPNWCNQGAGLGERPTVNP ***:***                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *
45	GH6_Ace CBHA_Cfi E3_Tfu	TDFPNAHLDAYVWIKPPGESDGTSAASDPTTGKSDPMCDPTYTTS--YGVLTN-ALPNS SGYAASHLDAFVWIKPPGESDGASTDIPNDQGRFDRMCDPTFVSPKLNQLTG-ATPNA ----APGVDAYVWVWIKPPGESDGASEEIPNDEGKGFDRMCDPTYQGNARNGNNPSGALPNA *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *
50	GH6_Ace CBHA_Cfi E3_Tfu	PIAGQWFPAQFDQLVANARPAV PLAGQWFEEQFVTLVKNAYPVI PISGHWFSAQFRELLANAYPPL *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *                   *

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**Example 3: GuxA includes a GH12 catalytic domain**

Sequence alignments and comparisons of the amino acid sequences of the *Acidothermus cellulolyticus* GuxA second catalytic domain (aa 860 to 1090), *Streptomyces* sp. 11AG8 cellulase 12A (endoglucanase) and *Streptomyces lividans* cellulase B (endoglucanase) polypeptides were prepared, using the ClustalW program (EMBL; supra). An examination of the amino acid sequence alignment of the GH12 domains indicates that the amino acid sequence of the GuxA second catalytic domain is homologous to the amino acid sequences of known GH12 family catalytic domains for *Streptomyces* sp. cellulase 12A and *S. lividans* cellulase B (See Table 4). The amino acid sequence predicted for the GuxA GH6 domain is approximately 45% identical to the *Streptomyces* sp. cellulase 12A GH12 domain and approximately 42% identical to the *S. lividans* cellulase B GH12 domain, indicating that the GuxA second catalytic domain is a member of the GH12 family (Henrissat et al. (1991), supra).

**Table 4. Multiple amino acid sequence alignment of a GuxA second catalytic domain and polypeptides with Glycoside Hydrolase Family 12 catalytic domains.**

20	Multialignment of related Glycoside Hydrolase Family 12 catalytic domain GH12_Ace: <i>Acidothermus cellulolyticus</i> GuxA Hydrolase Family 12 catalytic domain Cel12A_Ssp: <i>Streptomyces</i> sp. 11AG8 cellulase 12A(endoglucanase). GeneBank Acc. # AAF91283. CelB_Sli: <i>Streptomyces lividans</i> cellulase B (endoglucanase). GeneBank Acc. # AAB71950	
25	Cel12A_SSp CelB_SLi GH12_ACe	NQQICDRYGTITIQD-RYVVQNNRWGTSATQCINV-TGNG-FEITQADGSVPTN DTTICEPFGTTTIQG-RYVVQNNRWGTSAPQCVTA-TDTG-FRVTQADGSAPT CTPGPNQNGVTSVQGEYRVQTNEWNSSAQCLTINTATGAWTVSTANFSGGTG *.*:.*. . * *.**.*.:* *.**.. * .*: .: *: * *
30	Cel12A_SSp CelB_SLi GH12_ACe	GAPKSYPSVYDGYCHYGNCAPR-TTLEPMRISSIGSAPSSVSRYRTGNGVYNAAYDIWLDPT GAPKSYPSVFNGCHYTNCSPG-TDLFVRLDTVSAAPSSISYGFVDGAVYNASYDIWLDPT GAPATYPSIYKGCWGNCTTKNVGMPIQISQIGSAVTSWSTTQVSSGAYDVAYDIWTNST *** :***:.*.***: **:. . :****. :.:* :* * .....:***** :.*
35	Cel12A_SSp CelB_SLi GH12_ACe	PRTNG-VNRTEIMIWFNRVGPVQPIGSPVGT-AHVGGRSWEVWTGSNGSNDVISFLAPSA ARTDG-VNQTEIMIWFNRVGPVQPIGSPVGT-ASVGGRTWEVWSSGNGSNDVLSFVAPSA PTTGTQPNGTEIMIWLNSRGGVQPFQSGQTATGVTAGHTWNVWQGGQTSWKIISYVLTTPG . * * * *****: * :*:** ..* . *.:*:** * * : * :*: : ..
40	Cel12A_SSp CelB_SLi GH12_ACe	ISSWS-FDVKDFVDQAVSHGLATPDWYLTISIAGFEPWEGGTGLAVNSFSSAVN ISGWS-FDVMDFVRATVARGLAENDWYLTISVQAGFEPWQNGAGLAVNSFSSTVE ATSI SNLDLKAIFADAAARGSLNTSDYLLDVEAGFEIWQGGQGLGSSFSVSVT :. * :*: .: .:.* . ** .:***** :.* ** .*** :*

45

**Example 4: Mixed Domain GH6, GH12, CBD II, CBD III Genes and Hybrid Polypeptides**

From the putative locations of the domains in the GuxA cellulase sequence given above and in comparable cloned cellulase sequences from other species, one  
5 can separate individual domains and combine them with one or more domains from different sequences. The significant similarity between cellulase genes permit one by recombinant techniques to arrange one or more domains from the *Acidothermus cellulolyticus* GuxA cellulase gene with one or more domains from a cellulase gene from one or more other microorganisms. Other representative endoglucanase genes  
10 include *Bacillus polymyxa* beta-(1,4) endoglucanase (Baird et al, Journal of Bacteriology, 172: 1576-86 (1992)) and *Xanthomonas campestris* beta-(1,4)-endoglucanase A (Gough et al, Gene 89:53-59 (1990)). The result of the fusion of any two or more domains will, upon expression, be a hybrid polypeptide. Such hybrid polypeptides can have one or more catalytic or binding domains. For ease of  
15 manipulation, recombinant techniques may be employed such as the addition of restriction enzyme sites by site-specific mutagenesis. If one is not using one domain of a particular gene, any number of any type of change including complete deletion may be made in the unused domain for convenience of manipulation.

It is understood for purposes of this disclosure, that various changes and  
20 modifications may be made to the invention that are well within the scope of the invention. Numerous other changes may be made which will readily suggest themselves to those skilled in the art and which are encompassed in the spirit of the invention disclosed herein and as defined in the appended claims.

This specification contains numerous citations to references such as patents,  
25 patent applications, and publications. Each is hereby incorporated by reference for all purposes.

### Claims

1. A composition comprising a substantially purified thermostable GuxA peptide, said GuxA peptide comprising a first catalytic domain GH6, a second  
5 catalytic domain GH 12, a carbohydrate binding domain (CBD) type III, and a carbohydrate binding domain (CBD) type II.
2. The composition of claim 1 further defined as comprising a sequence of SEQ ID NO: 4, SEQ ID NO: 7, SEQ ID NO: 5 and SEQ ID NO: 8.
- 10 3. A thermal tolerant GuxA peptide having a sequence of SEQ ID NO: 1.
4. An industrial mixture suitable for degrading cellulose, such mixture comprising the GuxA polypeptide of claim 1.
- 15 5. The industrial mixture of claim 4 further defined as comprising a detergent.
6. An isolated polypeptide molecule comprising:
  - a) a sequence of SEQ ID NO: 4;
  - 20 b) a sequence of SEQ ID NO: 7;
  - c) a sequence of SEQ ID NO: 5;
  - d) a sequence of SEQ ID NO: 8;
  - e) a sequence of SEQ ID NO: 1; or
  - f) an amino acid sequence having at least 70% sequence identity with the  
25 amino acid sequence of a), b), c), d), or e).
7. A fusion protein comprising the polypeptide of claim 6 and a heterologous peptide.
- 30 8. The fusion protein of claim 7, wherein the heterologous peptide is a leucine zipper.



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9. A cellulase-substrate complex comprising the isolated polypeptide molecule of claim 6 bound to cellulose.
- 5 10. A vector comprising the polynucleotide molecule that encodes a polypeptide of claim 6.
11. A host cell genetically engineered to express the polypeptide molecule of claim 6.
- 10 12. A composition comprising the polypeptide molecule of claim 6 and a carrier.
13. An isolated antibody that specifically binds to the polypeptide molecule of claim 6.
- 15 14. A method for producing GuxA polypeptide, the method comprising:  
incubating a host cell genetically engineered to express the polypeptide molecule of claim 6.
- 20 15. A set of amplification primers for amplification of a polynucleotide molecule encoding GuxA, comprising:  
two or more sequences comprising 9 or more contiguous nucleic acids derived from the polypeptide molecule of claim 6.
- 25 16. A probe for hybridizing to a polynucleotide encoding GuxA, comprising:  
a sequence of 9 or more contiguous nucleic acids derived from the polypeptide molecule of claim 6.
17. An assay method for the detection of a polynucleotide encoding GuxA,  
30 comprising:

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amplifying a nucleic acid sequence with a set of amplification primers comprising two or more sequences of 9 or more contiguous nucleic acids derived from the polypeptide molecule of claim 6; and

correlating the amplified nucleic acid sequence with detected polynucleotide  
5 encoding GuxA.

18. A method for assessing the carbohydrate degradation activity of GuxA comprising:

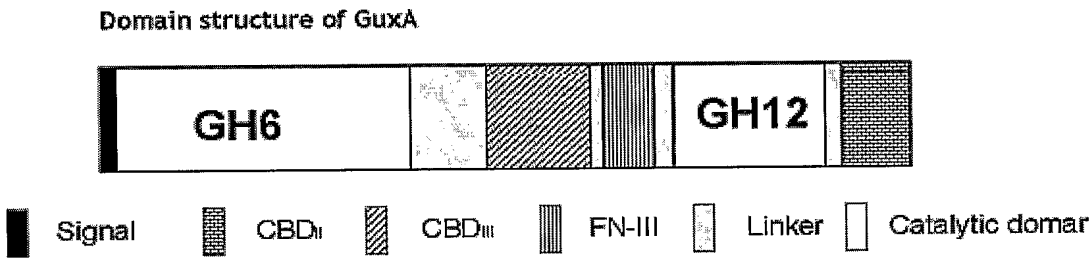
analyzing a carbohydrate degradation in the presence of GuxA and a  
10 carbohydrate degradation in the absence of GuxA on a substrate; and  
comparing the carbohydrate degradation in the presence of GuxA with the carbohydrate degradation in the absence of GuxA.

19. A method for reducing cellulose in a starting material, the method  
15 comprising:  
administering to the starting material an effective amount of a polypeptide molecule of claim 6.

20. The method of claim 19, wherein the starting material is agricultural  
20 biomass.

Fig. 1.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/23817

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/56 C12N9/42 C08B37/00 C12N15/62 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, EMBL

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MEINKE ANDREAS ET AL: "Cellobiohydrolase A (CbhA) from the cellulytic bacterium Cellulomonas fimi is a beta-1,4-exocellobiohydrolase analogous to Trichoderma reesei CBH II."</p> <p>MOLECULAR MICROBIOLOGY, vol. 12, no. 3, 1994, pages 413-422, XP008002307</p> <p>ISSN: 0950-382X</p> <p style="text-align: center;">--- -/--</p>	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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- \*P\* document published prior to the international filing date but later than the priority date claimed

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- \*&\* document member of the same patent family

Date of the actual completion of the international search

12 April 2002

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

In International Application No

PCT/US 01/23817

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SUNNA ANWAR ET AL: "A novel thermostable multidomain 1,4-beta-xylanase from 'Caldibacillus cellulosovorans' and effect of its xylan-binding domain on enzyme activity." MICROBIOLOGY (READING), vol. 146, no. 11, November 2000 (2000-11), pages 2947-2955, XP008002301 ISSN: 1350-0872 -----	
A	WO 00 14208 A (GENENCOR INT) 16 March 2000 (2000-03-16) -----	
A	WO 00 70031 A (ADNEY WILLIAM S ;BAKER JOHN O (US); SAKON JOSHUA (US); THOMAS STEV) 23 November 2000 (2000-11-23) -----	
A	WO 96 02551 A (MIDWEST RESEARCH INST) 1 February 1996 (1996-02-01) -----	

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Information on patent family members

In International Application No

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